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Viruses of symbiotic *Chlorella*-like algae isolated from *Paramecium bursaria* and *Hydra viridis*

(symbiosis/double-stranded DNA viruses/eukaryotic algae/pyrenoids)

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ABSTRACT **We previously reported that isolation of symbiotic *Chlorella*-like algae from the Florida strain of *Hydra viridis* induced replication of a virus (designated HVCV-1) in the algae. We now report that isolation of symbiotic *Chlorella*-like algae from four other sources of green hydra and one source of the protozoan *Paramecium bursaria* also induced virus synthesis. Algae from one of these hydra contained a virus identical to HVCV-1 (based on its rate of sedimentation, buoyant density, reaction to HVCV-1 antiserum, and DNA restriction fragments) whereas algae from the other three hydra contained another similar, but distinct, virus (designated HVCV-2). The virus from the paramecium algae (designated PBCV-1) was distinct from both HVCV-1 and HVCV-2. The symbiotic algae in the hydra could also be distinguished ultrastructurally. Chloroplasts of both algae that produced HVCV-1 lacked a pyrenoid whereas chloroplasts of the other three symbiotic algae contained pyrenoids. Since all symbiotic eukaryotic algae we have examined have had virus, a potential viral role in symbiosis is suggested.**

The fresh water hydroid *Hydra viridis* harbors a eukaryotic green *Chlorella*-like alga in a mutually beneficial symbiotic relationship (1). A characteristic number of these algae (ca. 10^5 per hydra) are located exclusively within cells of the gasterodermis of the hydra, where they carry out photosynthesis. Attempts to culture these algae free of the host have generally been unsuccessful, although Jolley and Smith (2) have reported culturing a *Chlorella* symbiont from a hydra isolated in England. All hydra strains, however, can be grown separately from the algae (termed aposymbiotic hydra) if provided with appropriate nutrients. An interesting property of the aposymbiotic hydra is that they retain the ability to rapidly reestablish association with symbiotic algae freshly isolated from green hydra. In contrast, algae not normally symbiotic with hydra are rejected by the hydra.

We recently found that a large (185 nm in diameter) double-stranded DNA virus (designated HVCV-1) began to multiply in the algae shortly after they were isolated from the Florida strain of hydra; replication of the virus resulted in lysis of the entire population of algae within 24 hr (3, 4). The appearance of the virus certainly impedes the culturing of this alga. The origin of the virus is unknown. It has not been detected in thin sections of algae in intact hydra, in algae immediately after isolation from the hydra, or in aposymbiont hydra (3).

We have examined *Chlorella*-like algae isolated from several sources of *Hydra viridis* and from the protozoan *Paramecium bursaria* to see whether they contain viruses and, if so, to determine whether they are similar to HVCV-1. The hydra were obtained from four commercial sources and one was isolated from a fresh water lake near Lincoln, Nebraska.

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MATERIALS AND METHODS

Source and Growth of Hydra and Paramecium. *H. viridis* was purchased from Connecticut Biological Supply, South Hampton, MA; Carolina Biological Supply, Burlington, NC; Wards Natural Science Establishment, Rochester, NY; and Sargent–Welch. Hydra were also isolated from Pawnee Lake near Lincoln, NE (designated Nebraska). The hydra, including the Florida strain, were grown in artificial pond water and fed daily on a diet of freshly harvested nauplii from *Artemia salina* as described (3). All five hydra strains were kept in a room separate from that used to grow the Florida strain and extreme care was taken to prevent contamination from the Florida strain.

P. bursaria were purchased from Carolina Biological Supply and grown on autoclaved oat seeds in artificial pond water (5).

Isolation and Properties of the Algae. Algae were isolated from the hydra and incubated in Bold's basal medium (6) as described (3). Algae were isolated from paramecia in the same way except that the protozoans were disrupted by ultrasonication (disruption was monitored by light microscopy). Algal lysis was measured by monitoring photosynthesis as described (3) except that tetracycline (100 μg/ml) was included to reduce bacterial growth. Separate experiments established that this concentration of tetracycline had no effect on photosynthesis of *C. vulgaris* over a 72-hr period. In some cases (when only a few hydra were used), the photosynthetic assay was scaled down to accomodate ca. 1×10^5 algae per assay. Samples examined by electron microscopy were processed as described (3).

Purification and Characterization of the Viruses. After the isolated algae had been incubated for 24–36 hr in Bold's basal medium, virus was purified by the HVCV-1 procedure (4). Procedures for determining the sedimentation coefficients in sucrose density gradients and buoyant densities in CsCl have been described (4). All of the virions contained a double-stranded DNA genome and isolation of the DNA, its treatment with restriction endonucleases, and electrophoresis of the DNA fragments have been described (4).

Serological Experiments. Antiserum was raised against purified HVCV-1 in randomly bred rabbits. The rabbits were given five intramuscular injections in the thigh (100–200 μg of virus in Freund's complete adjuvant per injection) at ca. 7-day intervals. The ability of the viruses to react with HVCV-1 antiserum was determined by the microprecipitin test (7).

RESULTS

Since the origin of HVCV-1 is not known, algae were isolated from all sources of hydra within hours after they arrived from the suppliers and were examined for photosynthetic activity. A rapid decrease in photosynthesis correlates with the appearance of HVCV-1 in algae from the Florida strain of hydra (3). As reported in Table 1 (experiment A), photosynthetic activity de-

Table 1. Photosynthetic activity of *Chlorella*-like algae isolated from several sources of hydra and from *P. bursaria*

		$^{14}CO_2$ incorporated, cpm per 30-min assay*						
		Source of the hydra						
Exp.	Time, hr	Florida	Wards	Sargent–Welch	Connecticut	Carolina	Nebraska	*P. bursaria*
A	0	25,000	39,000	45,000	28,000	28,000	35,000	—
	18	400	400	750	450	100	7,000	—
B	0	36,000	63,600	33,500	—	64,000	33,400	65,000
	18	700	570	200	—	800	2,000	40,000

Experiment A: With the exception of the Florida strain, assays were run with 1×10^5 algae immediately after receiving the hydra from the suppliers. Experiment B: The hydra were grown in the laboratory for ca. 3 months prior to assay, and 1×10^6 algae were used per assay.
* See ref. 3.

creased rapidly in algae (ca. 1×10^5) from all sources of hydra (five hydra per experiment). Hydra colonies from four of the sources, Carolina, Wards, Sargent–Welch, and Nebraska, were established (3–5 months) and photosynthesis was assayed with a larger (1×10^6) number of algae (experiment B). Again, photosynthesis rapidly decreased in algae from all sources of hydra. Likewise, photosynthesis decreased, but less rapidly, in algae isolated from paramecia. Furthermore, 0.1 M $MgSO_4$ delayed the decrease in photosynthesis in all algae. We had previously found that 0.1 M $MgSO_4$ delays lysis of algae from the Florida strain by HVCV-1 (unpublished data).

Ultrastructural Evidence for Virus in the Algae. Thin sections of intact hydra and paramecia and isolated algae were examined by electron microscopy. Electron micrographs of algae about 8 hr after their isolation from the Wards and Carolina hydra and from paramecium are shown in Fig. 1 *B*, *D*, and *E*, respectively. All samples contained polyhedral virus particles and were similar in size and morphology to HVCV-1. As noted previously for HVCV-1 (3), an intact nuclear membrane was never observed in algae with virus particles (Fig. 1 *B*, *D*, and *F*). Incubation of the algae for 24 hr results in lysis of the algae. Viral particles were not detected in thin sections of algae in intact hydra from any source (Fig. 1 *A* and *C*) nor in intact paramecia (Fig. 1*E*).

The algae from the various hydra could also be distinguished ultrastructurally. Algae from the Florida and Wards (Fig. 1*A*) hydra lack pyrenoids in their chloroplasts whereas algae from the Carolina (Fig. 1*C*), Sargent–Welch, and Nebraska hydra and from paramecia (Fig. 1*E*) contain pyrenoids.

Purification and Partial Characterization of the Viruses. All of the viral samples gave a single sharp A_{254nm} peak on sucrose density gradient centrifugation. The virus particles from algae from the Wards hydra sedimented at the same rate as HVCV-1 (ca. 2,580 S) whereas the particles from algae from Carolina, Sargent–Welch, and Nebraska hydra sedimented at ca. 2,300 S (Table 2). HVCV-1 and the virus from the Wards hydra gave a single sharp band at 1.295 gm/ml on buoyant density centrifugation. However, CsCl disrupted the viruses from the other hydra algae and from the paramecia algae. Purified viral particles from three of the algae, negatively stained with uranyl acetate, are shown in Fig. 1 *B*, *D*, and *F Insets*. The average sizes of the negatively stained particles are given in Table 2. The particles from the hydra algae fell into two size classes (170 nm or 180 nm) and the particles from the paramecium algae were slightly larger (ca. 192 nm).

The virus from the algae from the Wards hydra reacted with HVCV-1 antiserum to the same extent as did HVCV-1 (i.e., at a dilution of 1:512 of antiserum); in contrast, viruses from algae from the Carolina, Sargent–Welch, and Nebraska hydra and from paramecia did not react with HVCV-1 antiserum.

Analysis of the Nucleic Acid in the Virus Particles. The DNA from the Wards algal virus banded at the same density as HVCV-1 DNA [1.711 g/ml, 52% (G + C)] whereas the algal viral DNAs from Carolina, Sargent–Welch, and Nebraska hydra banded at 1.709 g/ml [50% (G + C)] (Table 2). The buoyant density difference between the HVCV-1 DNA and the other three viruses from hydra algae was significant since mixed DNA preparations separated into two bands on the gradients. The DNA from the paramecia algal virus banded at 1.704 g/ml [45% (G + C)].

Restriction endonuclease treatment of viral DNAs with *Bam*HI, *Sma* I, and *Hin*dIII produced characteristic fragments (Fig. 2). The DNA restriction patterns for HVCV-1 were identical to those for the viral DNA from Wards, and the patterns

Table 2. Properties of purified viruses and their nucleic acids isolated from *Chlorella*-like algae from several sources of hydra and from *P. bursaria*

	Source of the hydra					
Property	Florida	Wards	Sargent–Welch	Carolina	Nebraska	*P. bursaria*
Sedimentation coefficient of virus, S	2,580	2,580	2,300	2,300	2,300	2,300
Buoyant density of virus in CsCl, g/ml	1.295	1.295	ND	ND	ND	ND
Size of virus negatively stained with uranyl acetate, nm	170	170	180	180	180	192
Viral double-stranded DNA						
Buoyant density, g/ml	1.711	1.711	1.709	1.709	1.709	1.704
(G + C), %	52	52	50	50	50	45

ND, not determined (because virus from these algae were disrupted by CsCl).

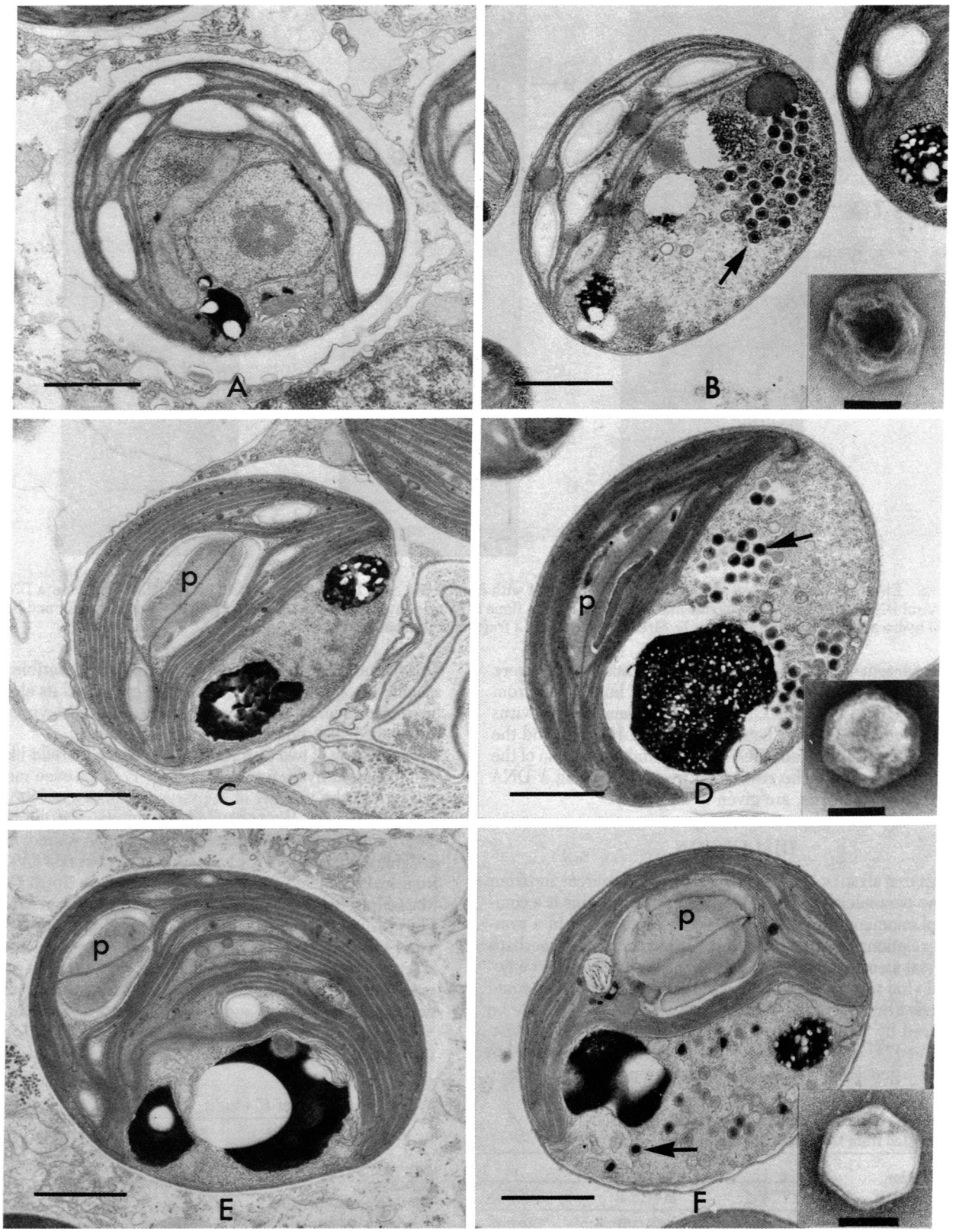

FIG. 1. Electron micrographs of the *Chlorella*-like algae present in (*A*) and 8 hr after isolation from (*B*) the Wards hydra, present in (*C*) and 8 hr after isolation from (*D*) the Carolina hydra, and present in (*E*) and 8 hr after isolation from (*F*) *P. bursaria*. Viral particles were present in all three algae 8 hr after isolation from the host (arrows). Note that the algae in the Carolina hydra (*C*) and in the paramecia (*E*) had a pyrenoid (p) whereas algae from the Wards isolate (*A*) did not. Bars = 1 μm. [*Insets* (*B*, *D*, and *F*)] Purified particles negatively stained with uranyl acetate. Bars = 100 nm.

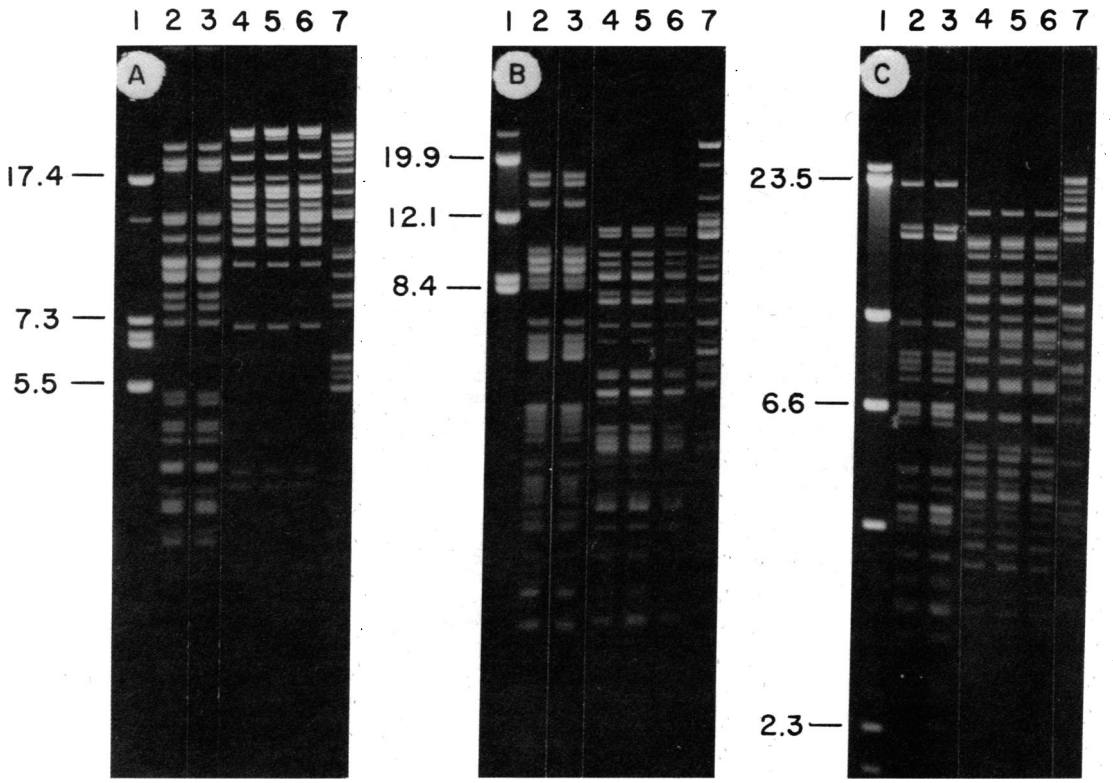

FIG. 2. Electrophoresis of algal virus DNAs after treatment with *Bam*HI (*A*), *Sma* I (*B*), and *Hin*dIII (*C*). The DNAs used were λ DNA (lane 1) and viral DNAs isolated from *Chlorella*-like algae from Florida (lane 2), Wards (lane 3), Carolina (lane 4), Sargent–Welch (lane 5), and Nebraska (lane 6) hydra and from paramecia (lane 7). Sizes of some of the λ fragments (kilobase pairs) are given.

for the Sargent–Welch, Carolina, and Nebraska viruses were identical to each other (designated HVCV-2) but distinct from that of HVCV-1 DNA. The DNA from the paramecia algal virus was distinct from those of both HVCV-1 and HVCV-2 and the virus has been designated PBCV-1. The number and sum of the molecular weights of the restriction fragments, using λ DNA fragments as standards, are given in Table 3.

DISCUSSION

The fact that algae isolated from hydras from all sources and from the one paramecia produced virus indicates that this is a common phenomenon for *Chlorella*-like algae that live in a symbiotic relationship with their host. The common and perhaps universal association of viruses with these algae suggests a potential viral role in these symbiotic systems. The viruses could be carried by the host and aid in the symbiont association or be produced by the symbiont to facilitate adaptation to the host. For example, the viruses could be lysogenic in the algae but express a gene(s) that alters the surface properties of the algae. This hypothetical gene product(s) could be responsible for the specific recognition between the host hydra and its algal symbiont. However, the exact role, if any, that these viruses play in the algal–host symbiotic process is unknown.

The finding of two distinct viruses in the *Chlorella*-like algae from different hydra was unexpected. Perhaps even more surprising was the identity of the viruses from a number of independent sources. We contacted the suppliers of the hydra to determine whether they came from a common source. As noted in Table 4, this was not the case. One algal virus (HVCV-2) came from hydra isolated from fresh water lakes in North Carolina, Massachusetts, and Nebraska. HVCV-1 virus (from Wards and Florida hydra) came from lakes in Massachusetts and Florida. The Florida strain of hydra has been maintained in culture for at least 20 years (R. L. Pardy, personal communication). Whatever their origin, the viruses probably have an ancient relationship with the algae and they are genetically stable.

The finding of two distinct algal viruses indicates that the viruses are unique to the hydra and not simply laboratory con-

Table 3. Molecular weights of the algal virus DNAs as calculated from the restriction fragments

Restriction endonuclease	$M_r \times 10^6$		
	HVCV-1	HVCV-2	PBCV-1
*Bam*HI	155 (34)	164 (18)	184 (29)
Sma I	155 (41)	113 (35)	186 (32)
*Hin*dIII	149 (36)	160 (34)	195 (32)

The M_r values for HVCV-1 DNA are ca. 10% higher than those published previously (4). Previously, a M_r of 30×10^6 was used for λ DNA; in these calculations, a value of 33×10^6 (taken from the Bethesda Research Laboratories catalog) was used. Numbers in parentheses are total numbers of restriction fragments observed.

Table 4. Sources of *H. viridis*

	State from which the hydra were collected	Virus type
R. L. Pardy*	Florida	HVCV-1
Wards Natural Science Establishment	Massachusetts	HVCV-1
Sargent–Welch†	Massachusetts	HVCV-2
Carolina Biological Supply	North Carolina	HVCV-2
Pawnee Lake, Lincoln, NE	Nebraska	HVCV-2

* This isolate was collected in Florida by M. H. Lenhoff and has been maintained in culture for ca. 20 years.

† These hydra were collected by the M. L. Taylor Corp. and sold to Sargent–Welch.

taminants (e.g., from the brine shrimp used to feed the hydra). However, the origin of the algal viruses is unknown. As discussed previously for HVCV-1 (3), HVCV-2 could be lysogenic in the algae or a few algal cells could be infected with virus (carrier state) that infect the remaining (some or all) algae on their release from the host. Regardless of the source of the viruses, their existence hampers the isolation and culturing of these algae. Furthermore, it appears to be advantageous for the algae to remain in a symbiotic relationship with the hydra so as to preclude lysis by the virus.

The source of PBCV-1 in the algae of *P. bursaria* is also unknown. We were unable to detect PBCV-1 in intact paramecium by electron microscopy and the virus appeared only after isolation and incubation of the algae. This is in contrast to a report by Kawakami and Kawakami (8), who described a virus infecting the *Chlorella*-like algae in a *P. bursaria* strain isolated in Japan. In that case, viral particles were readily detected both inside and outside the paramecium. Those viral particles, which were described only ultrastructurally, are similar in size and morphology to the algal viruses described here.

Heretofore, there have been few studies of viruses of eukaryotic algae (for review, see refs. 9, 10, and 11); one of the principal reasons has been the difficulty in obtaining sufficient quantities of virus. This difficulty is not as great a problem with these symbiotic algae since they lyse with reasonable synchrony. However, a major advance in studying these viruses would be the discovery of a way to infect free living algae. This would be significant because the viruses could be produced in larger quantities and possibly one could develop a biological assay. In this regard, PBCV-1 is especially promising because several investigators (ref. 12 and references cited therein) have cultured *Chlorella*-like algae from *P. bursaria*.

Note Added in Proof. Recently, we have examined *Chlorella*-like algae from two hydra, designated European and Coranation, obtained from D. C. Smith in England. Both algae produced an identical virus as judged by DNA restriction analysis (*Bam*HI and *Hin*dIII). These viruses were distinct from HVCV-1 and HVCV-2 and have been designated HVCV-3.

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